

EARLY T LYMPHOCYTES

Differentiation In Vivo of Adult Intrathymic Precursor Cells

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Although it is well accepted that immunocompetent T lymphocytes can be derived from blood-borne stem cells by a series of developmental events that occur within the mouse thymus (1, 2), the maturation sequence, inductive signals, and precursor-product or lineage relationships involved in this process are as yet poorly understood. An extensive literature suggests that cortical-type thymocytes are the precursors of the medullary-type thymocytes.¹ This conclusion is based on early data obtained from in vivo labeling experiments, as well as in vitro functional assays using isolated subpopulations of thymocytes, either in the presence or absence of added factors (for review, see Refs. 4–7). This concept has, however, been challenged by other in vivo labeling studies (8, 9), and more recently by in vitro functional assays using highly purified subpopulations, and by limiting-dilution analyses, leaving open the issue of the actual relationship between these two categories of thymocytes (10–12).

Several approaches have been taken to identify the intrathymic precursors to cortical and/or medullary-type thymocytes. The results of several studies on fetal thymic development in situ or in vitro organ cultures (12–21), or chimeras prepared with fetal thymus grafts (21), suggest that cells with cortical and medullary phenotypes are derived from early Thy-1⁺, Ly-1-low, Lyt-2⁻, L3T4⁻ thymocytes. In the most direct demonstration of a precursor-product relationship, Ceredig, et al. (19) showed that a proportion of purified Lyt-2⁻ fetal thymocytes will convert to Lyt-2⁺ after short-term in vitro culture. In addition, mature functional T cells (cytolytic and interleukin 2-producing) can be derived from organ cultures made from early fetal thymuses, which contain only Thy-1⁺, Lyt-2⁻, L3T4⁻ thymocytes, in the absence of any further influx of cells (16, 20).

Recently, we reported (21–24) a minor subpopulation of adult mouse thymocytes (<5%) that expresses low levels of Ly-1, and no Lyt-2 or L3T4 surface

¹ The terms cortical-type and medullary-type are used here to imply a surface phenotype (Lyt-2⁺, L3T4⁺ for cortical, and Lyt-2⁺, L3T4⁻ or Lyt-2⁻, L3T4⁺ for medullary, respectively) rather than an anatomical location. However, cells with these major phenotypes have been localized to the cortex and medulla in histological sections (3).

antigen. This subpopulation has been designated dLy-1,² for its dull staining with anti-Ly1 antibodies. The large size, blast-like morphology, and pattern of surface antigen expression of adult dLy-1 cells are similar to the predominant subpopulation found in day 15 fetal thymus. More extensive characterization confirmed that dLy-1 thymocytes are in the T lymphocyte lineage, and can be distinguished from cortical and medullary-type thymocytes, as well as from peripheral T cells by *in vivo* steroid sensitivity, agglutination with peanut lectin, and expression of surface antigens (24 and B. Fowlkes, L. Edison, B. Mathieson, and T. Chused, in preparation). dLy-1 thymocytes are enriched among regenerating thymocytes early after irradiation or *in vivo* steroid treatment (24), and among host cells repopulating thymus grafts (21, 22). These properties, taken together, suggest that dLy-1 thymocytes are early precursor thymocytes. More direct evidence of the precursor nature of these cells was provided by experiments in which 20–30% of isolated dLy-1 thymocytes differentiated to a Lyt-2⁺, L3T4⁺, or cortical phenotype in 20–24 h *in vitro* cultures (23).

In this report, we show that dLy-1 cells have a greater capacity for differentiation *in vivo* than *in vitro*. Using an assay system that allows subpopulation analysis of both host- and donor-derived cells, we show that isolated dLy-1 cells reenter the thymus of irradiated congenic mice and give rise to the other major subpopulations of thymocytes. These data suggest that dLy-1 cells are early intrathymic T cell precursors with multiple potential for differentiation, but with a limited capacity for self-renewal.

Materials and Methods

Mice. C57BL/6 (B6), B10.BR/SgSnJ, and C3H/HeN female mice (6–8 wk old) were obtained from the Small Animal Section, Veterinary Resources Branch, NIH. B6 mice, congenic for Ly antigens (bred from stock originally produced and provided by Dr. E. A. Boyse, Memorial Sloan-Kettering Cancer Center, NY) were maintained on an NIH contract to Bioqual Laboratories, Rockville, MD. The B6 Ly-congenic strains, C57BL/6-Ly-1^a (B6-Ly-1.1), and C57BL/6-Ly-2^aLy-3^a (B6-Lyt-2.1,3.1), possess the Ly-1.1 and Lyt-2.1,3.1 alleles, respectively (25). The B6-Ly-5.2-congenic strain possesses the Ly-5.2 allele (26).

Reagents. Mouse monoclonal antibodies (mAb) against Ly-1.2 (NEI-017), Ly-1.1 (NEI-003), Ly-5.1 (NEI-020), and Ly-5.2 (NEI-021) were obtained as ascites fluids from New England Nuclear, Boston, MA. Mouse mAb against Lyt-2.2 (clone 19/178), the gift of Dr. U. Hämmerling (27), was obtained from ascites or culture supernatants. Rat mAb to L3T4a, GK1.5 (28), was a gift of Dr. F. Fitch. GK1.5 was purified from culture supernatant by ion exchange and gel filtration or by affinity chromatography using goat anti-rat Ig (GARIG) coupled to agarose (62-9541; Zymed, San Francisco, CA). Purified antibody used for immunofluorescence was labeled with *N*-hydroxysuccinimidobiotin (Sigma Chemical Co., St. Louis, MO). Affinity-purified and biotin-labeled anti-Lyt-2 (clone 54-6.7) and anti-Ly-1 (clone 53-7.3) (29) were obtained from Becton-Dickinson Monoclonal Antibody Center, Sunnyvale, CA. Rat mAb against Thy-1.2 (J1j) (30) was obtained from Dr. J. Sprent. The mouse mAb, C3PO, against Ly-1.2 (31) was obtained from Dr. J. Klein. Dialyzed and sterile-filtered supernatant fluids of the latter two mAb were used for antibody and complement-mediated lysis with Lo-Tox-M rabbit complement (Cedar Lane Laboratories, Ontario, Canada). Affinity-purified second antibodies, goat anti-mouse Ig

² *Abbreviations used in this paper:* BM, bone marrow; dLy-1, dull-staining Ly-1; FC, flow cytometry; FITC, fluorescein isothiocyanate; GAMIG, goat anti-mouse Ig; GARIG, goat anti-rat Ig; HBSS, Hanks' balanced salt solution; mAb, monoclonal antibody; NMS, normal mouse serum; TR, Texas Red.

(GAMIg), goat anti-mouse IgG2 (GAMIgG2), and GARIg were raised and affinity purified in this laboratory, and labeled with fluorescein isothiocyanate (FITC) (Research Organics, Cleveland, OH) using standard procedures. GARIg was absorbed extensively until it was unreactive with mouse Ig. GAMIgG2 did not react with rat Ig. Affinity-purified avidin (from egg whites) was purchased from Sigma Chemical Co., and labeled with Texas Red (TR) (Molecular Probes, Inc., Junction City, OR) as described previously (32). Normal mouse serum (NMS), concentrated twofold by 50% saturated ammonium sulfate precipitation, was used for cold blocking in the two-color staining procedures described below.

Cell preparation. Thymocytes were obtained in single-cell suspension in cold Medium 199 (Gibco, Grand Island, NY) containing 5% fetal calf serum, as previously described (33). Bone marrow (BM) single-cell suspensions were prepared by standard procedures.

Isolation of subpopulations. dLy-1 thymocytes were isolated as reported previously (23) by a two-stage antibody and complement-mediated lysis procedure, using anti-Lyt-2.2, anti-L3T4, and anti-Ly-1.2 mAb. Yields were 10–30% of predicted values, with a purity of >97%. Two-color flow cytometric (FC) analysis of the purified population has been presented previously (23). Purity was also assessed by single-color fluorescence analysis using labeled, species-specific second antibodies.

Lyt-2⁺ or Lyt-2⁺ and L3T4⁺ thymocytes were isolated by a modification of previously described plate separation methods (34, 35). Briefly, appropriate amounts of mAb mouse anti-Lyt-2.2 or mouse anti-Lyt-2.2 plus rat anti-L3T4, titrated to give the highest yield and purity, were incubated with cells for 30 min at 4°C. After two washes, cells were transferred twice onto petri plates coated with titered GAMIg for selection of adherent thymocytes. Viability counts and FC analysis revealed that yields were 70–80%, and purity was >98% for Lyt-2⁺ or Lyt-2⁺ and L3T4⁺ cells.

To remove mature T cells, BM cells were treated with undiluted culture supernatant fluids containing anti-Thy-1.2 (J1j) and anti-Ly-1.2 (C3PO) plus complement, using the conditions described previously for thymocytes (23). Controls for all cytotoxic procedures included aliquots of cells with antibody alone, with complement alone, and with neither antibody nor complement.

Immunofluorescence Staining. For antibody binding, cells were adjusted to 2×10^7 cells/ml of phenol red-free, Hanks' balanced salt solution (HBSS) containing 0.1% bovine serum albumin and 0.1% NaN₃. Cells were incubated for 30 min at 4°C with a saturating concentration of antibody. After each incubation, cells were washed twice with 2 ml medium, and were resuspended in 0.3 ml of HBSS for FC analysis.

For single-color fluorescence, unlabeled or biotinylated antibody was used in the first incubation step, and labeled second antibody or avidin (FITC-labeled) was used for the second step, with washes between steps. For two-color fluorescence, the staining sequence was: (a) anti-Ly-1.1, Ly-1.2, Ly-5.1, or Ly-5.2; (b) FITC-labeled GAMIg; (c) NMS; (d) biotin-labeled rat anti-Lyt-2, -L3T4, -Ly-1, or combinations of these; and (e) TR-labeled avidin. The cells were washed after each incubation, except between the third and fourth incubations. NMS was used to block unoccupied binding sites on bound second antibody that had the capacity to react with both primary antibodies. Control thymocytes, either unstained or with second antibody alone, were used to obtain background fluorescence levels.

Cells stained for one-color fluorescence were analyzed for comparison with aliquots of the same cells doubly stained. Such controls were used to assess whether competition, crossreactions, or interactions occurred between reagents in two-color analyses. These controls were also used to establish whether spectra emission overlap occurred due to the combination of green and red fluorochromes used.

Flow Cytometry. FC was performed on a fluorescence activated cell sorter (FACS II; Becton-Dickinson, Mountain View, CA) as described previously (36). The data were stored and analyzed by a PDP 11/34 computer (Digital Equipment, Maynard, MA). All data were collected using three-decade log amplifiers and displayed on a log scale of increasing green and red fluorescence intensity, without defining units. 10^5 viable cells were analyzed for each two-color, and 5×10^4 cells for each single-color analysis. Dead cells were

excluded from analysis by forward light scatter (37) and propidium iodide staining by electronic gating (38).

Contour plots from dual-parameter analyses show increasing levels of cells in a 64×64 matrix. Each successive level represents a doubling of the number of cells per matrix point. To obtain percentages of thymocyte subpopulations, total counts were integrated in selected areas of the contour plots (as shown in Figs. 1–3). Single-parameter histograms were derived from dual-parameter analysis to facilitate this selection.

In the transfer experiments described, both Ly-1.2 and Ly-1.1 were analyzed for every thymus assayed. This was done as a control of each analysis for the other. It also allowed subpopulation analysis of both host- and donor-type subpopulations in the radiation chimeras. Since the expected complementary results were obtained, only results from analyses of Ly-1.2 are presented.

Radiation Chimeras. Recipient mice were given 750 rad from a Cs radiation source (Gammacell 40, Type B; Atomic Energy of Canada, Ltd., Ottawa, Canada). 15–24 h later, unfractionated or isolated populations of thymocytes or BM cells were used for intravenous transfer into irradiated recipients. Irradiated recipient mice were maintained on laminar flow racks in sterilized cages with sterilized food and bedding. The mice received water containing 2 g/l antibiotics (Biosol 325; Upjohn Co., Kalamazoo, MI) from 1 wk before irradiation until sacrifice.

Transfers were performed using cells from B6 (Ly-1.2) as donors to B6-Ly-1.1 recipients, from B6 (Lyt-2.2,3.2) donors to B6-Lyt-2.1,3.1 recipients, from B6 (Ly-5.1) donors to B6-Ly-5.2 recipients, or from B10.BR (Ly-1.2) donors to C3H/HeN (Ly-1.1) recipients. For kinetic studies, 5×10^6 cells were transferred into each mouse. Thymuses were removed and assayed for repopulation by two-color FC at multiple time points.

Results

Two-color FC Allows Delineation of Four Donor-derived Thymocyte Subpopulations in Radiation Chimeras. It is known that cortical-type thymocytes express both Lyt-2 and L3T4, while medullary-type thymocytes express high levels of Ly-1 (21, 39, 40), and Lyt-2 or L3T4, but not both (7, 10, 12, 21, 41). dLy-1 thymocytes express low levels of Ly-1, but neither Lyt-2 nor L3T4. In radiation chimeras constructed by transfer of thymocytes across an Ly-1 barrier, these four groups can be detected by serial examination for the coordinate expression of the donor Ly-1 allele with Ly-2, and for expression of Ly-1 with L3T4, as shown in Fig. 1. Two-color FC analysis for coordinate expression of Ly-1 and Lyt-2 delineates three populations of thymocytes (Fig. 1*a*). Analysis for coordinate expression of Ly-1 and L3T4 also demonstrates three populations (Fig. 1*b*). Taken together, these two types of two-color FC analysis detect four subpopulations of thymocytes: (I) dLy-1, Lyt-2⁻, L3T4⁻; (II) cortical-type, Lyt-2⁺, L3T4⁺; (III) medullary-type, Lyt-2⁻, L3T4⁺; and (IV) medullary-type, L3T4⁻, Lyt-2⁺ thymocytes. Selecting areas of contours that maximize separation of the four subpopulations gives the subpopulation distribution of: (I) 1–5%, (II) 85–90%, (III) 5–13%, (IV) 1–4% (range from 10 experiments; II is obtained by subtraction). Analysis for coordinate expression of Lyt-2 and L3T4 will reveal all four as separate subpopulations in a single analysis. However, neither of these antigens is expressed on all thymocytes, and alleles of L3T4 have not been demonstrated; therefore, analysis of Ly-1 in combination with each of these markers was necessary. Although it is not displayed in Fig. 1*c*, there is also a minor subpopulation of Ly-1-bright, Lyt-2⁻, L3T4⁻ cells that can be more easily detected among isolated Lyt-2⁻ thymocytes, and constitutes <1% of total thymocytes (21, 39, 41).

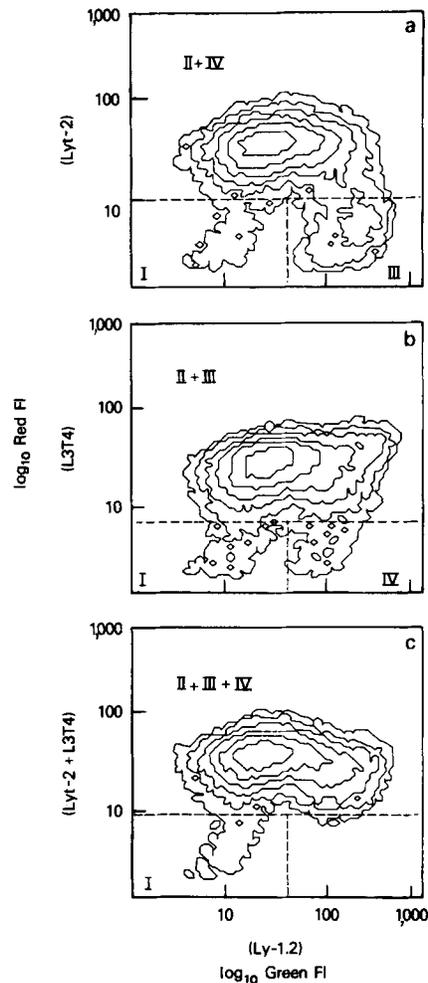


FIGURE 1. Coordinate expression by B6 thymocytes of Ly-1.2 and Lyt-2 (a), L3T4 (b), or Lyt-2 + L3T4 (c) delineates four subpopulations of thymocytes. Identity of subpopulations designated by Roman numerals: I, dLy-1 (Lyt-2⁻,L3T4⁻); II, cortical type (Lyt-2⁺,L3T4⁺); III, medullary type (Lyt-2⁻,L3T4⁺); IV, medullary-type (Lyt-2⁺,L3T4⁻). dLy-1 thymocytes express low levels of Ly-1, medullary-type express high levels (40), while cortical-type exhibit a broad range of Ly-1 expression. Analysis by two-color FC for coexpression by thymocytes of Ly-1 and Lyt-2 + L3T4 (c) demonstrates that the bulk of thymocytes that express high levels of Ly-1 are either L3T4⁺ or Lyt-2⁺. Staining procedures are described in Materials and Methods. Dotted lines indicate the regions of the contour used to obtain the fraction of each subpopulation. For these analyses the distributions are (a) I, 3.2%; II + IV, 89.7%; III, 7.1%; (b) I, 3.3%; II + III, 94.4%; IV, 2.3%; (c) I, 3.3%; II + III + IV, 96.0%.

To test the potential of purified dLy-1 thymocytes to reenter the thymus and differentiate *in vivo*, radiation chimeras were constructed using purified dLy-1 thymocytes for transfer into congenic hosts. There is a well-documented (42–44) transient rebound of intrathymic host cells after irradiation, making it necessary to distinguish host and donor thymocytes in the chimeras. By using B6-Ly-1.1 mice as recipients, and B6 thymocytes (Ly-1.2) as donors, host and

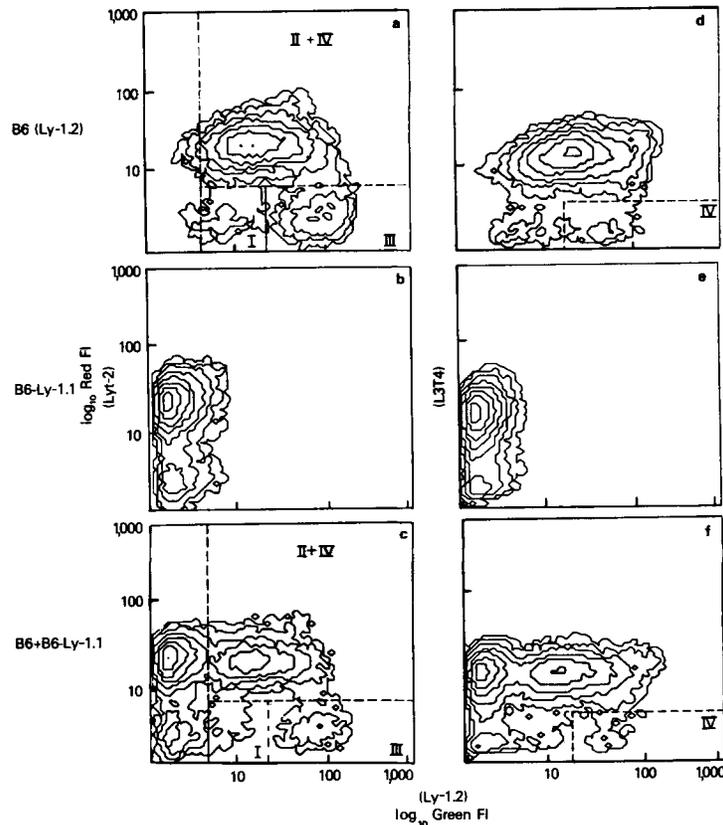


FIGURE 2. Two-color FC analysis of Ly-1.2 and Lyt-2 (*a-c*) or L3T4 (*d-f*) on artificial mixtures of thymocytes from B6 and B6-Ly-1.1 mice. All subpopulations except dLy-1 can be quantitated accurately. Reagents and procedures used are the same as those used in Fig. 1. Analyses are shown for B6 thymocytes, (*a, d*); B6-Ly-1.1, (*b, e*); and mixtures of B6 and B6-Ly-1.1 thymocytes (*c, f*). The dotted lines indicate the integration regions used to determine the subpopulation distributions given in Table II. To facilitate comparisons, Ly-1.2⁺ subpopulations are normalized to 100%. Subpopulations are identified in Fig. 1. From a representative experiment (analyzed as shown in the figure), the proportion of each Ly-1.2⁺ subpopulation for B6: I, 2.4%; II + IV, 86.9%; III, 10.7%; IV, 2.8% (total Ly-1.2⁺, 93.4%); for B6-Ly-1.1: (total Ly-1.2⁺, 1.8%); for B6 + B6-Ly-1.1: I, 3.5%; II + IV, 86.2%; III, 10.3%; IV, 2.7% (total Ly-1.2⁺, 54.9%).

donor subpopulations could, in principle, be distinguished by two-color FC analysis of Ly-1.2 or Ly-1.1 and Lyt-2 or L3T4. It was therefore important to establish the resolution and sensitivity of such a two-color FC assay for the detection of these subpopulations. This was done by analyzing artificial mixtures of host- and donor-type thymocytes, and comparing the results with those obtained from unmixed samples.

Thymocytes from B6, B6-Ly-1.1, and a mixture of these thymocytes were doubly stained and analyzed for Ly-1.2 and Lyt-2 (Fig. 2*a-c*) or Ly-1.2 and L3T4 (Fig. 2*d-f*). As shown, thymocytes from B6 mice were Ly-1.2⁺ (Fig. 2*a* and *d*), while thymocytes from B6-Ly-1.1 were Ly-1.2⁻ (Fig. 2*b* and *e*). Mixtures of these thymocytes resulted in contours that were additive (Fig. 2*c* and *f*).

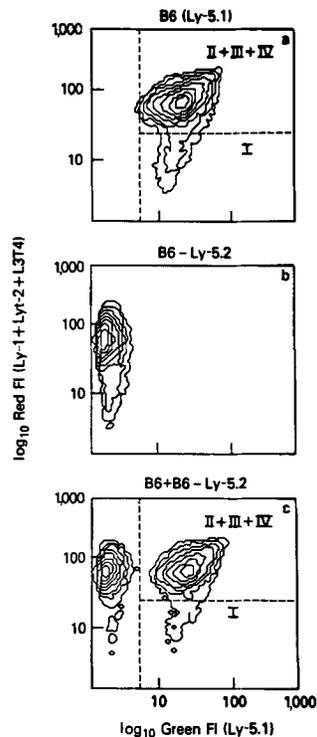


FIGURE 3. Two-color FC analysis of Ly-5.1 and Ly-1 + Lyt-2 + L3T4 on artificial mixtures of thymocytes from B6 (Ly-5.1) and B6-Ly-5.2 mice. In these mixtures B6 dLy-1 thymocytes (Ly-5.1⁺) can be discretely resolved from B6-Ly-5.2 dLy-1 thymocytes. Analysis made of: (a) B6, (b) B6-Ly5.2, and (c) mixture of B6 and B6-Ly-5.2 thymocytes. Dotted lines indicate integration regions used to obtain the distributions given in Table II. To facilitate comparisons, Ly-1.2⁺ subpopulations were normalized to 100%. Subpopulations are identified in Fig. 1. From a representative experiment (analyzed as shown in the figure), the proportion of each subpopulation for B6: I, 3.8%; II + III + IV, 96.2% (total Ly-5.1⁺, 99.6%); for B6-Ly5.2: (total Ly-5.1⁺, 0.6%); for B6 + B6-Ly-5.2: I, 3.9%; II + III + IV, 96.1% (total Ly-5.1⁺, 53.0%).

of contours were selected to maximize the separation between Ly-1.2⁺ and Ly-1.2⁻ subpopulations (Fig. 2). While the values for subpopulations II + IV, III, and IV in the mixtures were as expected, the values for I (dLy-1 cells) were imprecise. Despite the presence of Ly-1 antigen on all thymocytes, there is, nonetheless, overlap between Ly-1.2⁻ cells and Ly-1.2⁺ dLy-1 cells. This limitation of the Ly-1 congenic system was circumvented by using a second type of chimera, made by the transfer of isolated dLy-1 thymocytes from B6 mice into irradiated, B6-Ly-5.2 congenic mice. Ly-5 antigen (a determinant on the T200 molecule) is expressed at uniformly high levels on all thymocytes. Fig. 3a shows a contour plot of B6 thymocytes analyzed for coordinate expression of Ly-5.1 and Ly-1 + Lyt-2 + L3T4. Since dLy-1 thymocytes (I) express low levels of Ly-1, no Lyt-2 nor L3T4 antigens, but high levels of Ly-5, they can be accurately quantified by this procedure. The validity of this type of analysis is supported by the fact that the percentage of dLy-1 thymocytes obtained on aliquots of unfractionated thymocytes using either two-color FC analysis of Ly-5.1 and Ly-1 + Lyt-2 + L3T4 (Fig. 3a) or of Ly-1.2 and Lyt-2 (Fig. 1a) was the same. Furthermore,

TABLE I
 Donor-derived Thymocytes Recovered from B6-Ly-1.2 → B6-Ly-1.1 Radiation Chimeras as a
 Function of the Number and Type of Thymocytes Transferred

Thymocytes transferred	Donor-type (Ly-1.2 ⁺) on day 13 after transfer of thymocyte fraction:				
	Unfractionated	Lyt-2 ⁻	dLy-1	Lyt-2 ⁺	Lyt-2 ⁺ + L3T4 ⁺
			%		
5 × 10 ⁴	ND*	ND	0.2 ± 0.1	ND	ND
5 × 10 ⁵	0.2 ± 0.1	0.4 ± 0.2	2.2 ± 0.1	0.1 ± 0.0	0.3 ± 0.2
5 × 10 ⁶	0.8 ± 0.2	5.8 ± 0.2	21.1 ± 1.5	0.3 ± 0.1	0.4 ± 0.1
5 × 10 ⁷	7.8 ± 1.2	47.1 ± 4.2	ND	0.4 ± 0.0	0.2 ± 0.1

Mice received 750 rad 18 h before cell transfer. Values obtained by single-color FC analysis of Ly-1.2⁺ thymocytes from 3-4 individual mice. Unfractionated cells contain 3-4%, while Lyt-2⁻ thymocytes contain 20-30% dLy-1 cells. B6-Ly-1.1 unreconstituted radiation controls never exceeded 0.3% ± 0.1 Ly-1.2⁺. Mean ± SEM of the number of thymocytes recovered from recipient mice was 5.7 ± 0.4 × 10⁷. Unfractionated and Lyt-2⁻ thymocytes were treated with equivalent amounts of anti-Ly-1.2 antibody (without complement) to make them comparable to dLy-1 thymocytes. Thymocytes so treated were approximately fivefold less efficient in repopulation than untreated, unfractionated thymocytes, or unfractionated thymocytes treated with anti-Lyt-2, or anti-Lyt-2 + anti-L3T4.

* ND, not determined.

when artificial mixtures of B6 and B6-Ly-5.2 thymocytes were made and analyzed for coexpression of Ly-5 and Ly-1 + Lyt-2 + L3T4, the expected fraction of dLy-1 thymocytes was obtained in the mixture (using the selected areas integrated as shown in Fig. 3a). It is obvious however, that this system does not have the advantage of the B6 → B6-Ly-1.1 chimera, since it does not allow the visualization of other thymic cell types (II, III, IV) as discrete subpopulations.

Proportion of Donor-derived Thymocytes Is Directly Related to Number of Cells Transferred. Table I shows the proportion of donor-derived thymocytes in radiation chimeras after transfer of varying numbers of thymocytes of distinct phenotypes. The data indicate that the proportion of donor-derived cells in the thymus is directly related to the number of dLy-1 thymocytes used for transfer, whether unfractionated thymocytes, isolated Lyt-2⁻ thymocytes, or purified dLy-1 cells were tested. Most importantly, isolated Lyt-2⁺ (representing at least 85% of total thymocytes) or isolated Lyt-2⁺ + L3T4⁺ thymocytes (representing >94% of thymocytes) were completely unable to repopulate irradiated recipients. Even when 10⁸ Lyt-2⁺ thymocytes were transferred, no donor-derived thymocytes were detected (data not shown). These data strongly indicate that the cell responsible for thymocyte regeneration is Lyt-2⁻, L3T4⁻.

Donor-derived Cortical- and Medullary-type Thymocytes Are Detected after Transfer of dLy-1 Thymocytes in Radiation Chimeras. Two-color FC analysis was used to distinguish the donor-derived subpopulations generated by transfer of 5 × 10⁶ unfractionated thymocytes, dLy-1 thymocytes, or BM cells from B6 mice (Ly-1.2) into irradiated B6-Ly-1.1 mice. Recipient thymocytes were analyzed 7-31 d later. Fig. 4 shows typical results on thymocytes obtained from individual animals assayed 11 d after transfer. Thymocytes from an unirradiated B6 mouse are shown as a control. At this time point, almost no cells of donor-type (Ly-1.2) were detected when unfractionated thymocytes were used for transfer (Fig. 4b

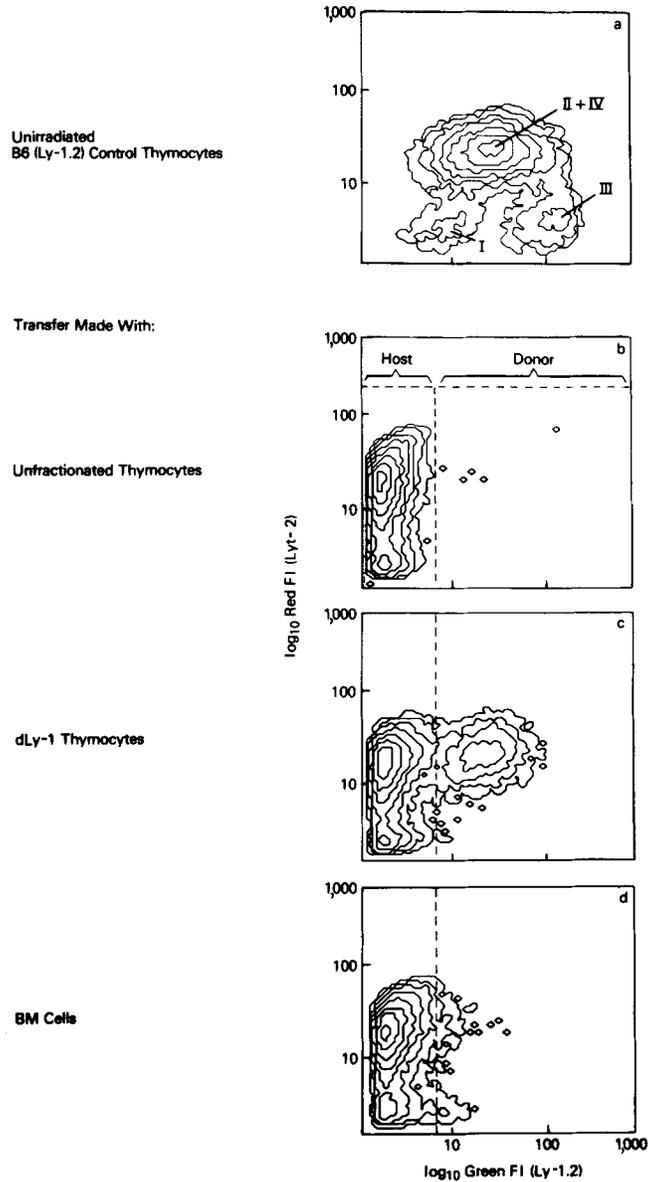


FIGURE 4. Thymocytes from B6 (Ly-1.2) → B6-Ly-1.1 radiation chimeras assayed by two-color FC analysis of Ly-1.2 and Lyt-2 on day 11 after transfer. At this time point, donor-derived thymocytes are readily apparent in the chimera made by dLy-1 thymocyte transfer. In contrast, only a few donor-type thymocytes are displayed in the BM chimera. (a) Thymocytes from unirradiated, normal B6 mice. Irradiated B6.Ly-1.1 were recipients of B6 unfractionated thymocytes (b), dLy-1 thymocytes (c), or BM cells (d).

and d); only a few were observed after transfer of BM cells. However, donor-derived cells were easily detected in the transfer made with dLy-1 thymocytes (Fig. 4c). Most importantly, Fig. 4c shows that most of the donor dLy-1 cells (I, the input subpopulation) had differentiated to Lyt-2-bearing cells.

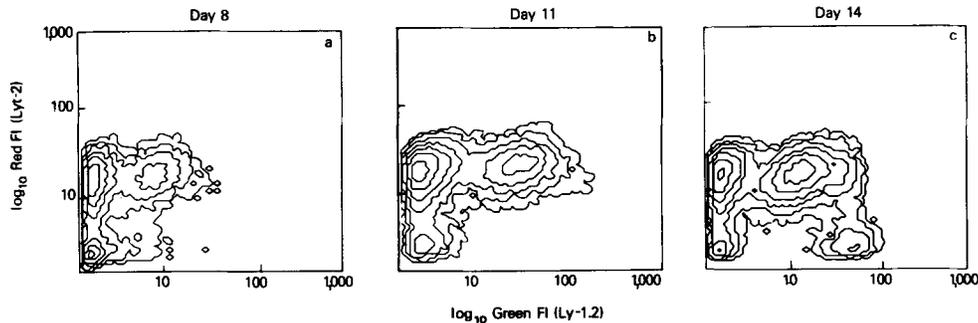


FIGURE 5. Thymocytes from B6 (Ly-1.2) \rightarrow B6-Ly-1.1 radiation chimeras assayed on (a) day 8, (b) day 11, or (c) day 14 after transfer of dLy-1 thymocytes. dLy-1 transfer results in the sequential appearance of donor-derived cortical (II) and medullary-type (III) thymocytes.

The sequence of differentiation of donor-derived thymocytes after transfer of dLy-1 cells is shown in Fig. 5. It can be seen that when dLy-1 cells were transferred, donor dLy-1 cells (I) were still detectable at day 8 (Fig. 5a), but were not present at day 11 (Fig. 5b). Donor-derived cortical-type cells (II) predominated at day 11. Subpopulation III, medullary-type thymocytes (Ly-1-high, Lyt-2⁻) appeared by day 14 (Fig. 5c). This donor-derived subpopulation (III) was also L3T4⁺, based on analysis of aliquots stained with Ly-1.2 against Lyt-2 + L3T4 (as shown in Fig. 1c). These analyses show that dLy-1 thymocytes differentiate to other thymocyte cell types in the radiation chimeras.

Donor-derived Thymocytes Are Detected Earlier after Transfer of dLy-1 Thymocytes than after Bone Marrow Transfer in Radiation Chimeras. The sequence of differentiation by donor-derived thymocytes was investigated. The kinetics of appearance of donor-derived thymocyte subpopulations for a series of transfers is presented in Table II. As a basis for comparison, the fraction of donor-derived thymocytes (Ly-1.2⁺) was normalized to 100%, and the percentage of each subpopulation was compared to the percentage of the same subpopulation in normal, unirradiated control B6 thymocytes analyzed on the same day. Analyses of Ly-1.2 and L3T4 indicate that before day 14, subpopulation IV, the Lyt-2⁺ medullary-type thymocytes, constituted <1% of the donor-derived thymocytes. As subpopulation IV became more apparent, aliquots were also stained with Ly-1.2 against Lyt-2 + L3T4, which verified that this subpopulation was indeed Lyt-2⁺. Moreover, in B6 \rightarrow B6-Ly-2.1,3.1 chimeras, made by transfer of dLy-1 thymocytes, this later-appearing subpopulation (IV) was Lyt-2⁺ by two-color FC analysis for coordinate expression of Lyt-2.2 and L3T4 (data not shown).

Thus, donor-derived subpopulations rose and fell in a sequential manner with the order: (a) dLy-1; (b) cortical-type Ly-2⁺, L3T4⁺; (c) medullary-type Lyt-2⁻, L3T4⁺; and finally (d) medullary-type Lyt-2⁺, L3T4⁻. The results indicate that dLy-1 cells, when transferred into irradiated recipients, give rise sequentially to the three other major thymic subpopulations. By day 19, medullary-type thymocytes (III and IV) were above control levels, while cortical-type thymocytes (II) were below. Moreover, the above normal levels of medullary-type thymocytes obtained at the later time points suggests a single transient wave of cells passing through the various stages of thymocyte maturation, without self-renewal.

TABLE II
Subpopulation Analysis of Donor-derived Thymocytes from Individual B6 (Ly-1.2) → B6-Ly-1.1 Radiation Chimeras Made by Transfer of dLy-1 Thymocytes or BM Cells

Days after transfer	Distribution of donor-derived (Ly-1.2 ⁺) subpopulations									
	dLy-1 thymocyte transfer					BM transfer*				
	Total percent donor	I	II + IV	III	IV	Total percent donor	I	II + IV	III	IV
7	9.4	23.4	75.6	1.0	0.4					
	8.1	11.1	87.2	1.7	0.4					
8	16.7	6.7	92.1	1.2	0.7	0.9				
	21.5	7.0	91.6	1.4	0.5	1.6				
11	30.3	3.3	94.4	2.3	0.4					
	20.9	3.8	94.3	1.9	0.6	9.4	28.7	69.2	2.1	0.1
	31.8	2.5	95.3	2.2	0.6	4.8	27.1	70.8	2.1	0.1
13	21.9	1.8	92.6	5.6	0.9					
	70.0	1.4	94.6	4.0	1.4					
14	35.5	2.1	89.6	8.3	1.4	27.1	7.9	90.4	1.7	1.1
	14.5	2.8	88.3	8.9	1.4	29.8	9.5	87.8	2.7	1.4
	23.0	2.6	83.5	13.0	3.0					
16	27.2	1.9	82.5	15.6	4.2	56.0	2.2	93.9	3.9	1.4
	13.5	2.9	78.4	18.7	5.5	65.3	3.3	92.2	4.5	1.8
19	15.7	1.3	77.0	21.7	5.1	77.4	1.6	89.8	8.6	2.5
	37.7	3.3	72.0	24.7	6.8	87.9	2.8	86.8	10.4	1.9
	17.1	0.2	57.2	42.6	14.0					
23	11.3	1.7	22.2	76.1	ND [‡]	95.1	1.8	88.9	9.3	2.4
						94.6	1.5	90.2	8.3	ND
31						96.4	2.9	88.4	8.7	2.2
						96.9	2.1	89.5	8.4	1.7
						94.4	4.5	86.4	9.1	2.3
Unirradiated	94.3	2.4	89.0	8.6	2.9					
B6 control [§]	±0.7	±0.1	±0.6	±0.5	±0.3					

B6-Ly-1.1 recipient mice received 750 rad 1 d before transfer of 5×10^6 cells/mouse. Values obtained by two-color FC analysis of Ly-1.2 and L3T4, and expressed as percent, obtained by integration of areas of contour to optimize discrimination of Ly-1.2⁺ and Ly-1.1⁺ thymocytes, as shown in Fig. 2. B6-Ly-1.1 radiation controls never exceed 1.7% Ly-1.2⁺. To facilitate comparisons, Ly-1.2⁺ subpopulations were normalized to 100%. Subpopulations designated as dLy-1 (I); cortical-type thymocytes (II); medullary-type Lyt-2⁻, L3T4⁺ (III); and medullary-type Lyt-2⁺, L3T4⁻ (IV).

* Although assayed, no appreciable number of donor-derived thymocytes were detected until day 11 after transfer of BM cells. BM chimeras at days 23 and 31 and B6 control mice are virtually 100% Ly-1.2⁺, but give values <100% due to integration used to optimize discrimination of host and donor-derived thymocytes (compare Figs. 1 and 2).

[‡] ND, not determined.

[§] Arithmetic means ± SEM of nine individual mice assayed on the same day as the radiation chimeras.

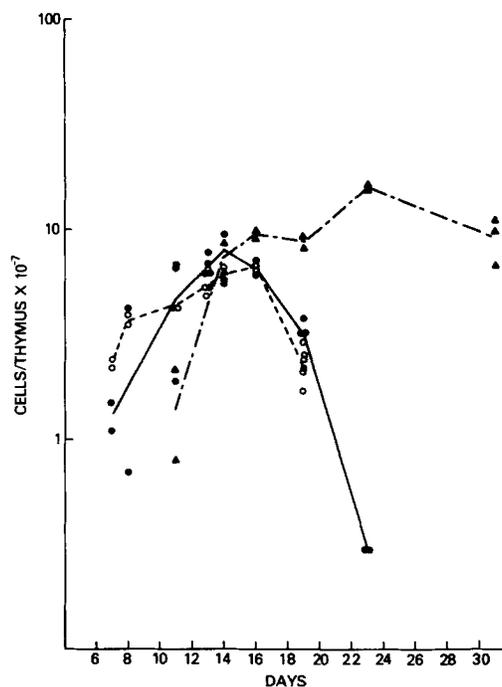


FIGURE 6. Regeneration of thymocytes in radiation chimeras made by transfer of dLy-1 thymocytes is transient. Total thymocyte recovery from radiation chimeras made by transfer of 5×10^6 unfractionated thymocytes (O), dLy-1 thymocytes (●), or BM cells (▲) is plotted as a function of time after transfer. While regeneration by thymocytes (host- and donor-type) is transient, repopulation by donor-type bone marrow is permanent. Each point represents cell recoveries obtained from individual thymuses. Lines are drawn through the geometric means determined for each time point.

The same order of subpopulation appearance was observed after BM transfer, but with a delay of 4–5 d. Although Table II indicates that donor-derived cells represent 94–96% of the total thymocytes at the latest time points analyzed after BM transfer, they actually are 100% of donor-type. This discrepancy is caused by selecting regions for integration that maximize resolution between host and donor (compare Fig. 2 with Fig. 1). Also, in contrast to dLy-1 transfers, at later time points after BM transfer (days 19–23), donor-derived subpopulations stabilized at control levels; i.e., there was no overshoot above control levels. Thus, it appears that BM transfer, in contrast to dLy-1 thymocyte transfer, results in a steady, renewable source of donor-derived thymocytes.

Repopulation by dLy-1 Thymocyte Transfer Is Transient. To more directly address this issue of transient vs. stable thymus repopulation, thymuses from mice receiving transfers of thymocytes or of BM were assessed in terms of total thymocytes recovered. Thymocyte recoveries from the radiation chimeras used in this series of transfers are shown in Fig. 6. In the results reported above, donor-type repopulation was always expressed in percent rather than absolute cell number. This was necessary to compensate for the variation in donor-derived

TABLE III
Subpopulation Analysis of Donor-derived Thymocytes from Individual B6 (Ly-5.1) → B6-Ly-5.2 Radiation Chimeras Made by Transfer of dLy-1 Thymocytes

Days after transfer	Total donor cells	Distribution of donor-derived (Ly-5.1 ⁺) subpopulations*	
		I	II + III + IV
		%	
5	17.3	17.8	82.2
	17.6	18.4	81.6
	16.0	18.3	81.7
7	16.8	7.7	92.3
	19.3	7.5	92.5
17	17.1	1.7	98.3
	14.7	1.5	98.5
	16.6	0.6	99.4
20	15.6	0.4	99.6
	21.1	0.5	99.5

B6-Ly-5.2 recipient mice received 750 rad 1 d before transfer of 5×10^6 of B6 thymocytes per mouse. Values obtained by two-color FC analysis of Ly-5.1 and Ly-1 + Lyt-2 + L3T4, expressed in percent obtained by integration of regions shown in Fig. 3. B6-Ly-5.2 radiation controls never exceeded 0.8% positive for Ly-5.1.

* To facilitate comparisons, Ly-5.1⁺ thymocytes were normalized to 100%. Subpopulations I-IV are identified in Table II.

repopulation. However, expression of the results in cell number does not alter the conclusions drawn from these data.

Since donor-derived repopulation with antibody-treated, unfractionated thymocytes was very low (Table I), thymocytes obtained from these recipients are derived principally from the rebound of host thymocytes after irradiation. Thymocyte recoveries from mice receiving unfractionated thymocytes or dLy-1 cells increased with time until day 13 or 14, and declined sharply after day 16. As described previously, mice receiving BM cells show a continuous increase in thymus cellularity due to donor repopulation, stabilizing after day 19 with 100% donor-type thymocytes, which are maintained indefinitely in reconstituted chimeras (43, 44). Thus, while the thymuses in irradiated mice are permanently restored by BM transfer, mice receiving dLy-1 thymocytes are only transiently repopulated. The possible cause of this difference is addressed below.

Donor-derived dLy-1 Thymocytes Show a Limited Capacity for Self-renewal. The finding that repopulation after dLy-1 transfer was transient suggested that dLy-1 cells, in contrast to BM cells, may have a limited capacity for self-renewal. To determine the persistence of donor-derived dLy-1 thymocytes in the radiation chimeras, dLy-1 cells isolated from B6 mice (Ly-5.1) were transferred into B6-Ly-5.2 irradiated recipients, and assayed at early and late time points using the type of analysis shown in Fig. 2. As presented in Table III, dLy-1 thymocytes of donor-type (I) decreased to background levels by day 20 after transfer. This result indicates that transferred dLy-1 thymocytes have a limited capacity for self-renewal in the irradiated host.

Discussion

This study shows that isolated dLy-1 thymocytes, when transferred into irradiated, congenic recipients, are able to reenter the thymus and transiently repopulate it with donor-derived cells. The proportion of donor-derived thymocytes obtained was a function of the number of dLy-1 thymocytes transferred. Moreover, the number of unfractionated or Lyt-2⁻ thymocytes of donor-type necessary for equivalent repopulation by dLy-1 was directly related to the proportion of dLy-1 thymocytes contained within these populations. In contrast, transfer of even very large numbers of purified Lyt-2⁺, or Lyt-2⁺ + L3T4⁺ thymocytes failed to generate any donor-derived thymocytes in irradiated hosts (Table I). Since these isolated thymocytes represent at least 95% of the total thymocytes, the results strongly indicate that an Lyt-2⁻, L3T4⁻ thymocyte is responsible for the donor-derived repopulation. dLy-1 thymocytes were able to generate both cortical and medullary-type thymocytes in the chimeras, demonstrating an even greater differentiation potential for these cells than had been obtained *in vitro* (23). Since the generation of donor-derived cells resulting from dLy-1 thymocyte transfer occurs over a 3-wk period, there is enormous opportunity for selection. However, we believe that the donor-derived medullary-type thymocytes cannot be the result of an expansion of medullary-type cells contaminating the dLy-1 cells used for transfer. We find that transfer of as many as 5×10^7 purified Lyt-2⁺ + L3T4⁺ thymocytes fails to generate any donor-derived thymocytes at day 16 when equivalent numbers of unfractionated control thymocytes (treated with anti-Lyt-2.2 and anti-L3T4 but not bound to plates) have produced both cortical and medullary-type thymocytes (data not shown).

The sequence of appearance of donor-derived subpopulations obtained after dLy-1 transfer was the same as that obtained after BM transfer, but occurred several days earlier. However, in BM chimeras, all donor-derived subpopulations stabilized at control levels between days 19–23 after transfer, whereas in dLy-1 chimeras, the proportion of donor-derived medullary-type thymocytes exceeded control levels. These findings, plus the observations that host thymocyte repopulation is transient, and that transferred dLy-1 cells exhibit a limited capacity for self-renewal (Table III), appear to account for the fact that total thymocyte numbers stabilize in BM chimeras but decline in chimeras made with dLy-1 thymocytes (Fig. 6). These data, taken together, suggest that BM transfers result in a steady renewable source of donor-derived thymocytes, whereas in thymocyte transfers, donor-derived dLy-1 thymocytes appear to differentiate to other cell types. Since these cells have a limited capacity for self-renewal, and since no earlier precursors are available to replace the dLy-1 cells, the donor-derived regeneration in chimeras made with thymocytes occurs as a single wave.

We have detected donor-type thymocytes as early as day 4 after transfer, the earliest time point at which enough thymocytes could be recovered from irradiated recipients for analysis. As shown here and elsewhere (43, 44), donor-type repopulation in BM chimeras is not detected until days 10–14. The reason that BM repopulation is delayed is unclear (45, 46). BM cells may seed the thymus only after establishing themselves in recipient marrow. Alternatively, BM pre-

cursors may go directly to the thymus but require additional time to obtain a status of differentiation comparable to dLy-1 thymocytes.

The fact that dLy-1 thymocytes are able to generate both donor-derived cortical- and medullary-type thymocytes in the chimeras also raises the issue of whether purification of dLy-1 thymocytes results in the coisolation of a blood-borne hemopoietic stem cell. However, the earlier and transient repopulation, and the limited self-renewal of dLy-1 cells distinguishes these cells from classically-defined BM stem cells. Instead, dLy-1 cells appear to be more differentiated than BM stem cells, since they produce no colony-forming units of spleen (21) or colony-forming units of culture after transfer into irradiated recipients (B. Mathieson, unpublished observations).

We interpret these findings from radiation chimeras as indicating that the dLy-1 cells represent the earliest T cell subset yet to be characterized. In support of this notion, we have been unable to observe any earlier subpopulation of T cells in the repopulation of thymus grafts, after *in vivo* steroid treatment, after whole-body irradiation, or in the developing fetal thymus (21, 22, 24). Moreover, we have shown that dLy-1 cells express β - but not α -chain of the T cell antigen receptor at the mRNA level (47). Thus, dLy-1 thymocytes may represent the earliest state of T cell maturation in the thymus. Others (45) have suggested that an intrathymic, self-renewing stem cell may exist; however, neither dLy-1 thymocytes nor large numbers of unfractionated thymocytes permanently reconstitute irradiated recipients (48, 49; see below). Therefore, if a self-renewing intrathymic stem cell exists, it is not able to home back to the thymus.

In two earlier studies (48, 49) using large numbers of unfractionated thymocytes ($5-10 \times 10^7$) for transfer, transient repopulation of the thymuses of irradiated mice by donor-derived cells was observed. The properties that were reported for these thymus-homing thymocytes appear similar to those dLy-1 thymocytes. Thymus-homing thymocytes were detected early after transfer (days 4-5), had a higher cortisone and radiation resistance than cortical thymocytes, and could not be detected at day 28 after transfer. Also, thymocytes from newborn mice showed a five times greater repopulation capacity (49). These findings are in agreement with our earlier observation (24) that dLy-1 cells occur in a greater proportion in newborns than in adults, and that these cells are highly enriched at early time points among regenerating thymocytes after radiation or *in vivo* steroid treatment.

Since there was also a transient repopulation by host-derived thymocytes in the radiation chimeras, it was necessary to selectively label donor-derived thymocytes. Two-color FC of Ly-1.2 plus Lyt-2 or L3T4, and of Ly-1.1 plus Lyt-2 or L3T4 allowed the detection of both donor and host-derived subpopulations. This system has several advantages over previously used chimeric systems. It permits the assay of a lymphoid surface antigen, Ly-1, that is present on all thymocytes (40). Antibodies to allelic forms of Ly-1 are available, so that host and donor thymocytes can be detected in a system unhampered by tissue rejection. There is no dilution or loss of label with time, as occurs after direct labeling with fluorescein or radioisotopes. Most importantly, this system allows detection of at least four subpopulations of thymocytes. Such resolution is not

possible from analysis of Thy-1.2 surface antigen (using Thy-1 congenic mice), even by two-color FC analysis, i.e., two-color FC analysis of Thy-1.2 and any other surface antigen that has been analyzed to date.

The data from the B6 → B6-Ly-1.1, and the B6 → B6-Ly-5.2 radiation chimeras, indicate that there is an ordered appearance of donor-derived subpopulations such that dLy-1 thymocytes (I) occur first, then cortical-type thymocytes (II), next L3T4⁺ medullary-type thymocytes (III), very closely followed by Lyt-2⁺ medullary-type thymocytes (IV). Kinetics studies have also been made using B6 → B6-Lyt2.1,3.1 and B10.BR → C3H chimeras (data not shown). Results from these chimeras are similar, except that in the former combination, only cortical-type (II) and medullary-type Lyt-2⁺ (IV) thymocytes (both are Lyt-2⁺) can be detected. This sequence of regeneration of subpopulations has been observed not only in the current systems using dLy-1 thymocytes or BM cells, but also in the regeneration of host-derived thymocytes in irradiated mice (regardless of whether they are reconstituted with transferred cells) (24). Moreover, we and others (12, 15, 18, 24) have observed this same sequence of appearance of subpopulations in the development of the fetal thymus.

As mentioned in Results, there is a minor subpopulation of thymocytes (<1%) that is Ly-1-bright and Ly-2⁻, L3T4⁻. Others (41) have speculated that this subpopulation is a precursor of more mature thymocytes. This subpopulation was not detected among the donor-derived thymocytes after transfer of dLy-1 thymocytes. If this minor subpopulation were not enriched at any time point after transfer, it would probably go undetected, since it would occur as <0.2% of the total thymocytes analyzed in the chimeras.

dLy-1 thymocytes have been defined and isolated by their lack of expression of Lyt-2 and L3T4, and low expression of Ly-1. However, a number of properties suggest that these cells may be heterogeneous. Only a proportion of these cells (20–30%) differentiate to cortical-type thymocytes in culture (23), dLy-1 thymocytes generate both cortical and two types of medullary-type thymocytes in radiation chimeras, and a proportion of hybrids made with dLy-1 thymocytes are not rearranged for the β -chain gene of the T cell antigen receptor (47). Furthermore, purified dLy-1 thymocytes are heterogeneous for expression of at least eight cell surface antigens (B. Fowlkes, L. Edison, and T. Chused, manuscript in preparation). Others (50, 51) have observed a heterogeneity of cell surface antigen expression within Lyt-2⁻, L3T4⁻ thymocytes. Whether such heterogeneity represents maturational differences, early commitment to more than one lineage, or is cell-cycle related remains to be determined.

In the radiation chimeras made with dLy-1 thymocytes, donor-derived medullary-type thymocytes were never observed in the absence of cortical-type thymocytes. In fact, in radiation chimeras made with either dLy-1 thymocytes, BM (this report), or in chimeras made with thymus grafts (21, 22), cortical-type always appeared before, or simultaneously with medullary-type thymocytes. Despite our failure to demonstrate medullary-type in the absence of cortical-type thymocytes, our experiments do not allow us to conclude that cortical-type thymocytes are the precursors of any or all medullary-type thymocytes. The chimeras made with purified dLy-1 thymocytes indicate that dLy-1 thymocytes

contain the precursors to the other major thymocyte subpopulations. The point at which the Lyt-2^+ and L3T4^+ medullary lineages diverge, i.e., in the BM or within dLy-1 or cortical thymocytes, has yet to be determined.

We and others (21, 24, 51) have pointed out the similarity between adult dLy-1 (or Lyt-2^- , L3T4^-) thymocytes and the predominant subpopulation that is found in the day 13–15 fetal thymus. These early fetal thymocytes are able, in fetal organ cultures, to generate both cortical- and medullary-type thymocytes with mature immune function, in the absence of any further influx of cells (16, 20). It seems reasonable to assume that adult thymocytes with the same phenotype would possess the same differentiation potential. However, while dLy-1 thymocytes generate donor-type cells with cortical and medullary phenotypes, these donor-derived cells have not, as yet, been tested for mature immune functions. Such an investigation is in progress. However, a high correlation has been shown (10–12) between medullary phenotypes and function. In this regard, we have recently determined that a fraction of donor-derived thymocytes in the chimeras express on their surface the T cell antigen receptor, as detected by an antibody specific for the β chain (52, 53).

In conclusion, our results suggest that adult dLy-1 thymocytes are currently the earliest recognizable T cells. Their phenotype is (as yet) indistinguishable from that of early fetal thymocytes. A precursor role is suggested for dLy-1 cells, since they are able to generate cortical-type thymocytes *in vitro*, and both cortical- and medullary-type thymocytes *in vivo*. These and other studies, which show that dLy-1 thymocytes express Ly-1 and Thy-1 (24), and mRNA for the β chain of the T cell antigen receptor (47) indicate the commitment of these cells to the T cell developmental pathway. Studies are in progress to determine whether this subpopulation contains cells in different states of maturation and/or cells already committed to more than one T cell lineage.

Summary

A minor subpopulation of adult murine thymocytes (<5%) that is Lyt-2^- , L3T4^- , and expresses low levels of Ly-1 (designated dLy-1 [dull] thymocytes) has been identified, isolated, and characterized. This study assesses the differentiation potential of dLy-1 thymocytes in the thymus *in vivo*. Using multiparameter flow cytometry, radiation chimeras of C57BL/6 mice congenic at the Ly-1 or Ly-5 locus, and allelic markers to discriminate host and donor, we showed that transferred dLy-1 cells were able to generate thymocytes expressing both cortical and medullary phenotypes in a sequential manner. The proportion of donor-derived thymocytes obtained was directly related to the number of dLy-1 thymocytes transferred. Transfer of purified Lyt-2^+ or $\text{Lyt-2}^+ + \text{L3T4}^+$ thymocytes, which constitute >94% of total thymocytes, failed to generate any donor-derived thymocytes in irradiated recipients.

Transfer of bone marrow (BM) cells produced the same sequential pattern of differentiation as that produced by dLy-1 cells, but was delayed by 4–5 d. Transferred dLy-1 thymocytes exhibited a limited capacity for self-renewal, and resulted in a single wave of differentiation in irradiated hosts. Thus, thymic repopulation by donor-derived cells after transfer of dLy-1 thymocytes was

transient, while repopulation by BM was permanent. These findings suggest that the isolated dLy-1 thymocytes described herein are precursor thymocytes that represent a very early stage in intrathymic development.

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